ISOLATION AND PARTIAL CHARACTERIZATION OF HUMAN ISOAMYLASES

by

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SUMMARY

Salivary isoamylases separate into 3 families of isoenzymes on gel filtration. The individual isoamylases of 2 of the families were isolated by isoelectric focusing. The isoenzymes showed extensive similarities in peptide pattern after cyanogen bromide cleavage and thermolysin digestion. They all shared common as well as minor specific antigenic determinants. Major enzyme activity was found in one carbohydrate free family possessing microheterogeneity. Another family having 20 per cent of total activity, showed a microheterogeneity due to varying sialic acid content. Differences in the number of neutral hexoses (0-15) and sialic acid residues resulted in a range of molecular weights (54 500-58 200) and isoelectric points (5.5-6.5). The minor third salivary isoamylase family comprising about 0.5 per cent of total activity, varied in sialic acid content and had isoelectric points 5.0-6.2.

The pancreatic group of isoamylases had a lower molecular weight (53 000) and contained no carbohydrate. They possessed great similarities to the salivary isoamylases in peptide pattern. The isoelectric points varied from 5.7 to 7.0. Their optimal enzymatic activity was at a slightly higher pH than for salivary isoamylases.

The group of isoamylases produced by the endometrium of isthmus uteri seemed to be closely related to salivary isoamylase. The fallopian tubes also produced these isoamylases as well as a specific group of tubal isoamylases having lower isoelectric points than any of the other groups of isoamylases. The tubal specific isoamylases varied in their sialic acid content.

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INTRODUCTION

Non-identity between human salivary and pancreatic isoamylases (E.C. 3.2.1.1.) was first detected by Nørby (1) who found salivary and pancreatic isoamylases to have separate electrophoretic mobilities. Muus & Vnenchak (2) first demonstrated multiple forms of salivary isoamylase. These early studies have since been amply confirmed in a number of reports (for review see ref 3) and some information is now available on the properties of the various isoamylases. Using ordinary electrophoretic techniques the normal salivary isoamylase is separated in 3 major as well as some additional minor isoamylase fractions. The normally occurring salivary isoamylase has been divided into 2 families of isoenzymes differing in gel filtration pattern and carbohydrate content but not in their amino acid composition (4,5). Also for the pancreatic group of isoamylases some physico-chemical properties have been determined (6). Few studies have been performed on isolated isoamylases from these 2 sources (7) or on the isoamylases produced by the female genital tract (8,9,10).

The present work was undertaken to study structural differences between isolated isoenzymes of the most common phenotype of both salivary and pancreatic isoamylases (about 90 per cent in a Swedish population) as well as to give some further information of the genital isoamylases[1].

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[1]Foot note: In this paper the following denotation of the various isoamylases is used. The salivary group of isoamylase is separated into 3 families, the A-, B-, and C-family; the individual isoenzymes within the B- and C-families are numbered from 1 to 3 according to their electrophoretic migration rates towards the anode. The individual isoamylases of pancreatic origin are called P_1-, P_2-, and P_3-isoamylase.

MATERIAL AND METHODS

Reagents

Sodium dodecyl sulfate (SDS), dansyl chloride, trifluoroacetic acid, polyamide layer sheets, N,N,N',N'-tetramethylenediamine, and N,N'-methylenebisacrylamide were obtained from BDH Chemicals Ltd (Poole, England), N,N'-methylenebisacrylamide was used after recrystallisation from chloroform; neuraminidase was purchased from Behringwerke (Mahrburg-Lahn, Germany); Biogel® from Bio-Rad Laboratories (Richmond, California); acrylamide (No 5521X) from Eastman Kodak Co (Rochester, New York); ethylenimine from Fluka AG (Buchs, Switzerland); human serum albumin from Kabi (Stockholm, Sweden); Ampholines® from LKB (Stockholm, Sweden); thermolysin from Merck AG (Darmstadt, Germany); agarose, batch 269A from Miles Seravac (Maidenhead, England); dithiothreitol from Nutritional Biochemicals (Cleveland, Ohio); Phadebas® Amylase Test and Sephadex® from Pharmacia (Uppsala, Sweden); phenylisothiocyanate (sequenal grade) from Pierce (Rockford, Illinois); cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin, and guanidine hydrochloride (ultra pure) from Schwarz Mann (Orangeburg, New York). Human transferrin was available at the laboratory. Rabbit anti-human total parotid amylase which also precipitated pancreatic and genital isoamylases was prepared as previously described (11). All other chemicals were reagent grade obtained from commercial sources.

General procedures

Agarose gel electrophoresis was performed in 0.075 M barbital buffer, pH 8.6 containing 2 mM calcium lactate as described by Johansson (12). The gels were stained for protein with Coomassie Brilliant Blue. For specific identification of the isoamylases an immunofixation technique using rabbit anti-human parotid isoamylase was applied (11). The isoamylases of samples having low enzyme concentration were identified by staining for isoamylase activity with the aid of the dye-starch polymer Phadebas Amylase Test (13). Prior to electroimmunoassay (14) 0.6 per cent linear polyacrylamide polymer was incorporated in the agarose gel to reduce electroendosmosis (13). After electrophoresis the relative amounts or activities of the individual isoamylase frac-

tions were determined by densitometric scanning using a Skalar Densiscan KS 3 (Kipp & Zonen, Delft, Holland) or by visual estimation.

Polyacrylamide disc gel electrophoresis was performed according to Davies (15) but omitting the spacer and sample gels. Thin-layer electrofocusing in polyacrylamide gel, with or without subsequent crossed electrophoresis in antibody containing gel, was done as earlier described (16,17).

Isoamylase preparation

Salivary isoamylases - Parotid saliva from two persons with the most commonly occurring salivary isoamylase phenotype was collected by means of a cup (18) placed over the orifice of Stensen's duct. After centrifuging, the supernatant was frozen at -20°C at least over night. After thawing, saliva from several collections were pooled and the saliva was recentrifuged at 10 000 g for 15 minutes. The supernatant was dialyzed at 4°C against 0.02 M tris-buffer pH 7.4 containing 0.15 M sodium chloride. After recentrifugation the supernatant was ultrafiltered and concentrated about 10 times with a DC2 Amicon hollow fiber dialyzer concentrator (Amicon Corp. Lexington, Mass.) furnished with an HIDP 10 filter cartridge retaining molecules greater than 10 000 daltons. Fifteen ml portions of the concentrate were gel filtered on a 5 x 88 cm Sephadex G-100 column at 4°C using the tris-buffer containing sodium chloride for elution. The flow rate was 55 ml per hour. After appropriate pooling of the fractions according to their isoamylase content as determined by agarose electrophoresis, the pools were concentrated about 100 times using the Amicon equipment. The pools were then dialyzed against 0.13 M glycine over night.

Further preparation of the individual isoamylase fractions was achieved by isoelectric focusing (19) (LKB, Stockholm, Sweden) using a sucrose gradient (0-40 per cent) in a column containing ampholytes at a final concentration of 1 per cent. The proportions of the individual Ampholine ranges pH 5-7 and 6-8 were 4:1. The electrode solutions were 0.15 M H_2SO_4 and 0.06 M NaOH, the column was prepared with the anode at the top. The separation was carried out at 4°C for 24 h at 300 V followed by 540 V until 48 h. The column was eluted in about

60 fractions. The isoamylase pattern of each individual fraction was determined by agarose electrophoresis.

Preparative flat-bed electrofocusing with Sephadex G-75 as stabilizing medium (20) was adopted using the Multiphor system (LKB, Stockholm, Sweden). The amylase pools from the gel filtration step were adjusted to 95 ml with distilled water before the addition of 5 ml ampholyte mixture. After the addition of 5 g Sephadex G-75 Superfine excess water was evaporated reducing the slurry to 75 per cent of its original weight (20). The separation was performed at 10°C for 2 h at 150 V followed by 300 V for another 15 to 17 h. The amylase zones were identified after protein staining of a heat dried (110°C) filter paper reprint of the gel surface. The relevant zones of the gel were collected and the isoamylases were separated from the ampholytes by placing the corresponding gel slurry on top of a Bio-Gel P10 column (2.4 x 40 cm) which was equilibrated with 3.4 M acetic acid. The acetic acid in the pooled fractions were removed in a rotary evaporator followed by freeze-drying. The freeze-dried isoamylases were used for determination of neutral hexoses and hexoseamines.

Pancreatic isoamylase - Pancreatic juice was obtained from patients undergoing pancreatic surgery by means of a catheter inserted in the main pancreatic duct. Specimens were also collected by cannulation of the papilla of Vater in connection with endoscopic retrograde cholangiopancreatography. The patients all belonged to the most common pancreatic isoamylase phenotype. The material was stored at -20°C until preparation of the individual isoenzymes. The preparation was performed essentially as described for the salivary isoamylases.

Genital isoamylase - The material used was comprised of endometrial washings obtained with jet wash technique using 0.15 M sodium chloride and of homogenates of genital tissue obtained at operations (total hysterectomy because of genital cancer). Thus samples from the fallopian tube and the endometrium of isthmus of the same individuals could be collected. After careful rinsing in 0.15 M sodium chloride the tissue specimens were homogenized with a Turmix tissue homogenizer in 0.25 M phosphate buffer pH 7.0 containing 0.15 M sodium chloride. After homogenization the samples were sonified and centrifuged at 4 000 g for

15 minutes. The supernatant obtained was used for further analysis after concentration. The jet washings were used merely after centrifugation and concentration. Gel filtration was performed on a Sephadex G-100 column with Blue Dextran, albumin, and cytochrome c as internal markers. The isoamylase pattern was detected by enzymatic staining after electrophoretic separation.

Methods for characterization of isoamylases

The isoelectric points of the isoamylases were determined by electrofocusing in the 110 ml Ampholine column. The pH of the fractions were determined within 30 minutes at 4°C by means of a Radiometer pH Meter 26 (Copenhagen, Denmark). The isoamylase pattern of the fractions was electrophoretically detected with the immunofixation technique and the amount of amylase was determined with electroimmunoassay. For each group of isoamylases, with exception of gel filtration pool-A (Fig. 1A) at least 3 determinations were performed and the means calculated.

For molecular size determinations polyacrylamide gel electrophoresis in SDS was performed essentially according to Weber and Osborn (21) with gel slabs containing 5, 7.5,and 10 per cent acrylamide. Human transferrin, ovalbumin, and chymotrypsinogen were used as molecular weight markers and cytochrome c as an internal reference. The molecular sizes reported represent the means of at least 5 individual determinations.

The NH_2-terminal amino acid residues of the B_3-, C_3- and P_3-isoamylases were determined using a modified (22) Dansyl-Edman technique with SDS in the coupling buffer followed by chromatography on polyamide sheets (23).

Carbohydrate analysis - For hexosamine determination the isoamylase was hydrolyzed for 6 h in 4 M hydrochloric acid at 100°C in evacuated and sealed tubes. Hexosamines were determined on a long column of the amino acid analyzer with a program for plasma or urine amino acids (24).

The total amount of neutral hexose of the various isoamylases was determined with the anthrone method as described by Spiro (25).

Two to four determinations were performed for each isoenzyme. Galactose was used as standard.

Determination of fucose was performed by means of the Dische-Shettles cysteine-sulphuric acid reaction as described by Spiro (25).

Determination of sialic acid using the thiobarbituric acid assay of Warren (26) was done after previous hydrolysis at 80°C for 1 h with 0.05 M sulphuric acid.

Neuraminidase treatment - About 0.5 mg isoamylase prepared from column electrofocusing was dissolved in 1 ml 0.1 M sodium acetate buffer pH 5.6 containing 1 mM calcium chloride or a solution containing isoamylase was dialyzed against this buffer for 24 h at 4°C. Various amounts, from 0.2 to 25 µl neuraminidase (500 U/l) were added to 100 µl of the isoamylase solutions. They were incubated at 37°C for one h and subsequently at room temperature over night. The neuraminidase treated samples were analysed by staining for protein or isoamylase activity after agarose gel electrophoresis or thin layer gel electrofocusing.

Cyanogen bromide cleavage of the purified isoamylases was performed in 70 per cent formic acid with 200 molar excess of cyanogen bromide (27). After incubation for 24 h at room temperature the samples were evaporated, diluted with water and subsequently lyophilized. This procedure was also used for neuraminidase degraded isoamylases. After cyanogen bromide treatment thin layer electrofocusing was performed in polyacrylamide gel containing 7 M urea pH gradient 3.5-10 (16). Before application of the sample in 10 M urea solution, the gel was prerun at 150 V for 1 h. The separation was performed at 150 V for 1 1/2 h followed by 500 V for 15 h.

After electrofocusing the gel was immersed in a fixation solution containing 0.65 M trichloracetic acid and 0.2 M sulfosalicylic acid. The gel was washed in 3 successive baths of 7.5 per cent acetic acid before being stained for carbohydrates using periodic acid Schiff (PAS) reagent (28). After photographic documentation of the carbohydrate containing fragments the gel was restained for protein by use of Coomassie Brilliant Blue.

Peptide mapping - Reduction and aminoethylation of the various isoamylases were performed essentially as described by Slobin & Singer (29)

using Tris-HCl pH 8.6 buffer containing 0.01 M EDTA and 7 M guanidine hydrochloride. Ethylenimine was added in 10 fold molar excess to dithiothreitol (DTT). The solution was extensively dialyzed against water at 4°C and subsequently lyophilized.

Thermolysin digestion was performed on 5 mg reduced and aminoethylated protein, dissolved in 1.2 ml 0.1 M ammonium hydrogen carbonate. Thermolysin 0.1 mg in 200 µl 0.1 M ammonium hydrogen carbonate containing 1 mM calcium chloride was added. After incubation for 3 h at 37°C another 200 µl thermolysin solution was added and the mixture was further incubated for 3 h. During the incubation period the mixture was regularly shaken. The sample was lyophilized twice.

For peptide separation the protein digest was dissolved in pH 6.4 electrophoresis buffer (pyridine:acetic acid:water, 25:1:225 by vol.). About 1 mg was applied to a Whatman 3 MM filter paper. High voltage (2.5 kV) electrophoresis was performed in a Varsol-cooled (+10°C) tank (Electrophoretic apparatus Model D, Gilson Medical Electronics, Middleton, Wisconsin) for 65 min. Subsequent ascending chromatography was performed in pyridine:isoamyl alcohol:water (35:35:30 by vol.) for 15 h (30). After drying the chromatogram a 4 cm broad strip was cut from the paper. The strip contained the zone of those peptides being neutral at pH 6.4 (localized by a ninhydrine stained guide strip from the electrophoretic separation). The strip was stitched to a new sheet of Whatman 3 MM paper for a second electrophoretic separation at pH 1.9 in acetic acid:formic acid:water (4:1:45 by vol.) for 55 min at 3.0 kV. The peptide maps were stained with cadmium-ninhydrine reagent and developed at room temperature.

Antigenic relationship - Antisera against the salivary isoamylases of pool-B, pool-C, and the pancreatic P_3-isoamylase were raised in rabbits by duplicate injections of about 1 mg of the corresponding antigens emulsified in complete Freund's adjuvant. Double radial immunodiffusion was performed according to Ouchterlony (31) and the corresponding electrophoretic analysis technique, immunoelectromigration was performed as described by Grubb (32). Isolated isoamylase as well as solutions containing the 2 groups of female

genital tract isoamylases were investigated. In the latter case no detectable precipitation arcs were produced, therefore staining for enzyme activity after conventional electrophoretic separation was used to disclose crossreaction.

pH optimum - The routine method for the determination of amylase activity in the present work was that using the dye-starch polymer Phadebas Amylase Test. This reagent table contains an insoluble coloured dye-starch polymer as well as buffer salts to give final concentrations of 10 mg/ml for the starch polymer, 0.05 M sodium chloride, 0.02 M sodium azide and 0.02 M phosphate buffer pH 7.1 in a 4 ml sample. To determine the pH dependence of the various isoamylases 25 tablets were suspended in 100 ml water and aliquots of this dispersion were adjusted using either 2 M HCl or 2 M NaOH to various pH values between 6.2 and 8.6 at intervals of 0.3 pH units measured at 21°C. By the use of a magnetic stirrer a homogenous dispersion was obtained from which 4 ml was pipetted into separate test tubes which were equilibrated at 37°C for 5 minutes before 100 µl of sample solution was added. After 30 minutes the reaction was stopped by the addition of 1 ml 0.5 M NaOH. The absorbance of the supernatant obtained after centrifugation at 10 000 g for 10 minutes was read in a Zeiss PM Q II spectrophotometer at 610 nm. Double tests were used and blanks were obtained by the addition of 0.15 M sodium chloride solution instead of sample solution. The sample solutions used were freshly prepared dilutions of the isolated isoamylases in 0.15 M sodium chloride. The dilutions used were selected to give an absorbance of about 0.7 at the pH of maximal activity.

RESULTS

Isoamylase preparation

Salivary isoamylase - Fig. 1A illustrates the gel filtration pattern of salivary isoamylase. Pool A comprised less than 0,5 per cent of the total isoamylase activity. This pool contained a number of isoamylases which separated in four groups on electrofocusing. Agarose electrophoresis showed a further separation into 9 fractions (Fig. 2A).

The second pool, comprising the B-family was made up of 3 fractions, B_1, B_2, and B_3 where B_1, is the most anodal fraction (Fig. 2). These comprised about 1, 9,and 10 per cent, respectively, of total activity. The last pool eluted from gel filtration, family-C, also separated into subfractions, C_1, C_2 and C_3, (Fig. 2) covering 0,5, 2 and about 77 % of total activity. The relative contribution of the various isoamylase showed negligable variation between different samplings. Owing to minimal amounts of some fractions, only B_1, B_2, B_3, C_2,and C_3 were further studied.

Pancreatic isoamylase - The pancreatic isoamylases had the same elution volume on Sephadex G-100 as pool-C of the salivary isoamylases (Fig. 1B). Using electrofocusing it was possible to further separate the fractions P_1, P_2,and P_3 (Fig. 3A and B) with 1, 9, and 90 per cent of the total isoamylase activity. They had a higher pI and migrated more slowly to the anode on agarose electrophoresis as compared with salivary isoamylases.

Genital isoamylase - The isoamylases from endometrium of isthmus had an elution pattern on the Sephadex G-100 column corresponding to pool-C of salivary isoamylases with the same electrophoretic mobility of the subfractions. The isoamylases of tubal origin separated into 2 families on gel filtration corresponding to the pool-B and pool-C of the salivary isoamylases (Fig. 1C). The 4 separate fractions corresponding to the B-pool had a higher electrophoretic mobility than corresponding fractions in saliva (Fig. 3C).

Partial characterization of isoamylases

Isoelectric points - The results are given in Table I and Fig. 2. The B_3- and C_3-isoamylases had identical pI:s as evident from both types of electrofocusing. However,agarose electrophoresis at pH 8.6 shows a clear separation of the two fractions. The pI:s of pancreatic fractions were 7.0, 6.4, and 5.7. The isoamylases of endometrium had pI identical with the salivary B-isoamylases but the specific fractions of the tubal homogenates had pI:s being less than 5.5 (Fig. 3D).

Molecular size determination with SDS-polyacrylamide electrophoresis on gels with 5, 7.5,and 10 per cent acrylamide all gave identical results

of individual isoamylases in the range of 52.700-58.200 daltons as shown in Table I. Using reduced samples no evidence for subunits were found.

No free N-terminal amino acids were found using the combined Dansyl-Edman method using SDS in the system. The procedure was run for the two first amino acids of the isolated isoamylases with lysozyme as control.

Carbohydrate analysis - The amount of total neutral hexoses corresponded to 15, 10, and 7 residues for the B_1-, B_2-, and B_3-isoamylases, respectively. The salivary pool-C and pancreatic isoamylases contained less than 1 per cent neutral hexose, which was interpreted as contaminants from the preparation steps. No glucosamine was found in the P_3- and C_3-isoamylases; the B-isoamylases contained 2 residues of glucosamine as shown in Table I. Fucose could not be demonstrated. Sialic acid was qualitatively found in the B_1- and B_2-isoamylases. The B_3-, C_2-, and C_3-isoamylases as well as all the P-isoamylases were asialoproteins.

After neuraminidase treatment the salivary pool-A and pool-B isoamylases behaved similarly. They focused as single fractions with higher pI corresponding to the most cathodal A-isoamylase and B_3-isoamylase, respectively. The pI:s and electrophoretic mobilities of the various salivary pool-C isoamylases and the pancreatic isoamylases were not influenced by neuraminidase treatment (Fig. 4). The electrophoretic mobilities of the anodal isoamylases of the endometrium of isthmus changed to that of the cathodal main isoamylase of that source, the migration rate of which was not influenced. The electrophoretic migration rates of the far anodal migrating specific tubal isoamylases were step-wise retarded by neuraminidase treatment to that of the slowest migrating tubal specific isoamylase.

Cyanogen bromide cleavage of the B_3-isoamylase resulted in 11 polypeptides as detected by protein staining after thin layer electrofocusing (Fig. 5). The 3 most intensely stained polypeptides as well as another weak polypeptide were also Schiff positive. The native B_1- and B_2-isoamylases contained 2 fragments not being present in native B_3-isoamylase. These 2 fragments stained more intensely for carbohydrate in B_1- than in the B_2-isoamylase. The pattern of CNBr-

fragment changed for neuraminidase treated B_1- and B_2-fractions to that of untreated B_3. The P_2- and P_3-isoamylases had identical polypeptide patterns after cyanogen bromide cleavage. Eleven separate polypeptides were demonstrable, 7 of which had isoionic points identical with polypeptides of the salivary isoamylases, while the remaining 4 polypeptides from the pancreatic isoamylases did not correspond to those from the salivary isoamylases. None of the C- or P-isoamylase polypeptides were stained with the Schiff reagent.

Peptide mapping - After thermolysin digestion and electrophoresis at pH 6.4, about 30 ninhydrin positive spots could be detected on the anodal part of the paper (Fig. 6A). On the cathodal side about 40 spots were visualized (Fig. 6C) and the electrophoretic separation at pH 1.9 disclosed about 40 additional peptides among the material being neutral at pH 6.4 (Fig. 6B).

The salivary isoamylases produced almost identical peptide maps. Two neutral peptides (marked 1) were present in the C_2- and C_3-isoamylases but were not clearly detectable in the B-isoamylases. Four of the acid peptides of the salivary isoamylases were absent in the P_3-isoamylase (vertically lined in Fig. 6). The pancreatic isoamylase contained one acid peptide not being present in any of the salivary isoenzymes (horizontally lined in Fig. 6). Of the neutral peptides 6 were specific for the salivary isoamylases while 2 (one of which was heavily stained) were specific for the pancreatic isoamylase. Three moderately basic peptides were found exclusively in the salivary isoamylases. One rather heavily stained strongly basic peptide was specific for the pancreatic isoamylase.

Antigenic relationship - All isoamylases treated, showed common antigenic determinants being responsible for most of their antigenic activity. However, specific antigenic sites were found in the following cases. Each of the B-isoamylases was found to contain one weak individual and one weak family specific antigenic determinants. The C_2-isoamylase contained one antigenic determinant not being present in C_3. The pancreatic isoamylases all contained antigenic determinants not being present in any of the salivary isoamylases. In addition the P_3-isoamylase had an antigenic determinant not being present in

P_2 or P_1 and the P_2-isoamylase had one not occurring in P_1.

The antiserum produced after immunization with the salivary pool-B did not show any distinct quantitative or qualitative differences from that produced with the pool-C. The precipitate of the pancreatic P_3-isoamylase on quantitative electroimmunoassay was blurred when any of the antisera against salivary isoamylase were used. The precipitate was also proportionally higher than when anti-P_3 serum was used. The precipitates produced by the salivary B_3- and C_3-isoamylases were blurred and proportionally higher when the anti-P_3 serum was used (Fig. 7A). Thus, immunization with the P_3-isoamylase produced an antiserum containing a proportionally higher titre of antibodies against pancreatic isoamylase than against salivary isoamylases. Compared to C_3, the B_3-isoamylase resulted in a proportionally higher precipitate when antiserum produced against pancreatic isoamylase was used than when antisera against any of the salivary isoamylases were used.

Antisera raised against any of the 2 families of salivary as well as against the pancreatic isoamylases alo precipitated the 2 types of isoamylases produced by the female genital tract (Fig. 8B).

pH optimum - All the salivary isoamylases had their maximum starch degrading activity at pH 7.4 while the pancreatic isoamylases all had their maximum activity at pH 7.7.

DISCUSSION

Due to the wellknown fact that amylase is abnormally retarded on Sephadex gel filtration it was easily separated from other large molecules being present in the sample. The low molecular weight substances of the original sample were eliminated by the concentration steps. A partial separation of the salivary isoamylases into 3 families of enzymes facilitated the following isolation of the individual isoenzymes. Isoelectric focusing was the further purification step used for the isolation of the isoamylases. As amylase has affinity for sucrose from the gradient of the electro-

focusing column, electrofocusing in flat bed was preferable. To remove the ampholytes and eliminate the unspecific absorption to the gel matrix acetic acid was used in the desalting gel filtration step. The present relatively simple method for the isolation of the individual isoamylases yielded isoenzymes of satisfactory purity for further studies.

Most physico-chemical studies of human isoamylases (for review of early reports, see 3) have been performed on partly separated salivary isoamylases (4,5) corresponding to the present B- and C-families although in two studies individually isolated salivary isoamylase fractions have been used (7,33). It has been shown that the salivary isoamylases can be separated into 2 families on gel filtration, one containing about 5 per cent carbohydrate and one without carbohydrate but concerning content of sialic acid conflicting results are reported (4,5,7,33). The molecular weights have been stated to be 55 000 to 60 000 daltons for the C- and B-families, respectively (5,7). No significant differences in amino acid composition are reported between the 2 families (4) or between individual salivary isoamylases (7). A close antigenic relationship has been reported for the 2 families of salivary isoamylase and the pancreatic isoamylase (33,34 and see 3).

According to the present study family A- and B-isoamylases contain carbohydrates and different amount of sialic acid which may explain the microheterogeneity within these families. The microheterogeneity within the C-family cannot be explained from our results.

As the human isoamylases all shared common antigenic sites and have great correspondence in polypeptide pattern after thermolysin digestion and cyanogen bromide cleavage, it can be concluded that they are closely related. The relationship was strongest between the families of salivary isoamylases where no significant difference in the protein part of the salivary pool-B and pool-C isoamylases could be demonstrated.

Unfractionated pancreatic amylase was earlier stated to contain no carbohydrate; to have a molecular weight of about 54 000 daltons and to possess great similarities to the salivary isoamylases in amino acid composition (6).

We also found the pancreatic group of isoamylases to have a somewhat lower molecular weight than the salivary group and to contain no carbohydrate. They possessed great similarities with the salivary isoamylases in the pattern of polypeptides obtained after cyanogen bromide cleavage and thermolysin digestion. They had their optimal enzymatic activity at a slightly higher pH than the salivary isoamylases, 7.7 and 7.4,respectively. The presence of individual pancreatic isoamylases was not due to differences in sialic acid content.

An interesting observation related to the occurrence of multiple pancreatic isoamylases has been made where isoamylases having the electrophoretic migration rates of the P_1- and P_2-isoamylases increase proportionally more than do the P_3-isoamylase in cases with obstruction of the pancreatic duct system (35). A possible reason may be charge differences due to deamidation of asparagine and glutamine residues as suggested for cytochrome c (36,37). In cases with obstructive pancreatic disease the amylase will be in proteolytic enzyme environment during a longer period than normally and thus possibly be subjected for proteolysis.

The isoamylases produced by the endometrium of isthmus seemed to be closely related to salivary isoamylase. These isoamylases were also produced by the fallopian tubes together with a specific group of tubal isoamylases having lower isoelectric points than any of the other groups of isoamylases. The tubal specific isoamylases also showed variations in their sialic acid content as seen after neuraminidase treatment.

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FIGURE LEGENDS

Fig. 1. Amylase elution patterns on Sephadex G 100 gel filtration. The arrows represent from left to right the elution volumes of Blue Dextran, albumin, and cytochrome c. The peaks represent amylase activity. A:Salivary isoamylase; B:Pancreatic isoamylase and C:Tubal isoamylase.

Fig. 2. Salivary isoamylases. A:Identified with enzymatic staining technique after agarose gel electrophoresis. Sample 1 parotid saliva, samples 2-5 pool-A isoamylases separated with isoelectric focusing and subjected to electrophoresis in agarose, sample 6 pool-B isoamylases and sample 7 pool-C isoamylases. B:Protein staining after polyacrylamide gel electrophoresis. Sample 1 pooled salivary amylase, sample 2 B_3-isoamylase, sample 3 B_2-isoamylase, sample 4 B_1-isoamylase, sample 5 C_3-isoamylase, and sample 6 C_2-isoamylase preparation. Isoelectric points are given for pool-A isoamylases.

Fig. 3. A:Pancreatic isoamylase identified with immunofixation technique after agarose gel electrophoresis. B: Protein staining after polyacrylamide electrophoresis. Sample 1 pooled pancreatic amylase, sample 2 P_3-isoamylase, sample 3 P_2-isoamylase, and sample 4 P_1-isoamylase preparation. C:Isoamylases identified with enzymatic technique after agarose gel electrophoresis. Sample 1 serum showing C_3-(above) and P_3-isoamylase, sample 2 homogenate of fallopian tube, and sample 3 homogenate from endometrium of isthmus. D:Enzymatic staining after thin layer isoelectric focusing. Sample 1 serum, sample 2 pancreatic juice, sample 3 saliva, sample 4 uterine washing, and sample 5 homogenate of fallopian tube.

Fig. 4. Effect of neuraminidase treatment on various isoamylases. Neuraminidase treated samples are indicated with N.

Fig. 5. Cyanogen bromide fragments of various isoamylases. Neuraminidase treated samples are indicated with N. A:Stained for protein, B:Same separations stained for carbohydrate.

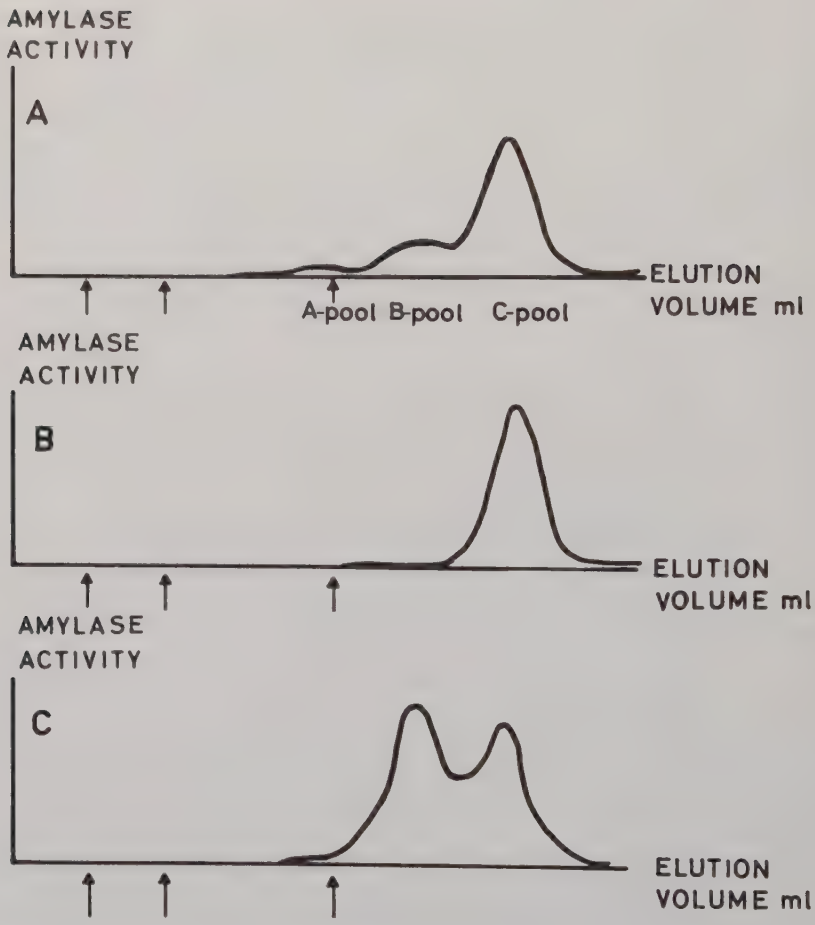
Fig. 6. Peptide mapping obtained by thermolysin digestion. Electrophoresis was in the horizontal direction; chromatography in the vertical direction. A:Acid peptides at pH 6.4. B:Neutral peptides at pH 6.4 reseparated at pH 1.9. C:Basic peptides at pH 6.4. Unfilled symbols represent weakly stained peptides, crosshatched symbols moderately stained peptides, blackened areas heavily stained peptides, vertically lined symbols peptides present only in salivary isoamylases, and horisontally lined symbols represent peptides present only in pancreatic isoamylase. Peptides marked 1 were present in the C-isoamylases, but weak or absent in the B-isoamylases.

Fig. 7. A:Precipitates obtained on electroimmunoassay. The same sample solutions consisting of the B_3-, C_3-, and P_3-isoamylases were subjected to electrophoretic migration in gels containing antisera against the B_3-, C_3-, and P_3-isoamylases, respectively. B:Electrophoretic separation of genital isoamylases stained for enzyme activity. Sample 1 rabbit antiserum, 2 and 3 homogenates of fallopian tube, 4 and 5 homogenates of the endometrium of isthmus. Samples 3 and 5 were preincubated with antiserum against parotid amylase.

Table I

Physico-chemical characteristics of isolated isoamylases

	Isoamylase					
	B_1	B_2	B_3	C_2	C_3	P_3
Isoelectric point	5.5	5.9	6.5	5.9	6.5	7.0
Molecular weight	58 200	57 800	57 200	54 800	54 500	52 700
Glucoseamine	2	2	2	0	0	0
Neutral hexoses	15	10	7	0	0	0
Sialic acid	+	+	0	0	0	0
Mol weight, apoprotein	54 700	54 800	55 000	54 800	54 500	52 700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ACTIVITY
A
ELUTION
VOLUME ml
A-pool B-pool C-pool
AMYLASE
ACTIVITY
B
ELUTION
VOLUME ml
AMYLASE
ACTIVITY
C
ELUTION
VOLUME ml

Fig. 1

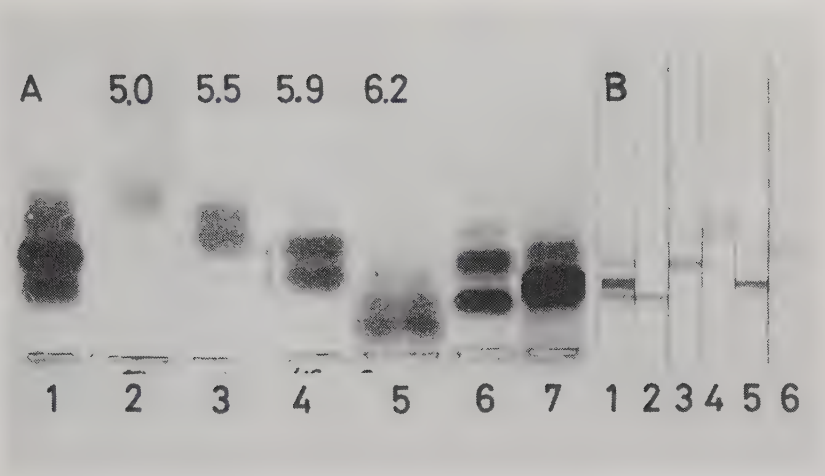


Fig. 2

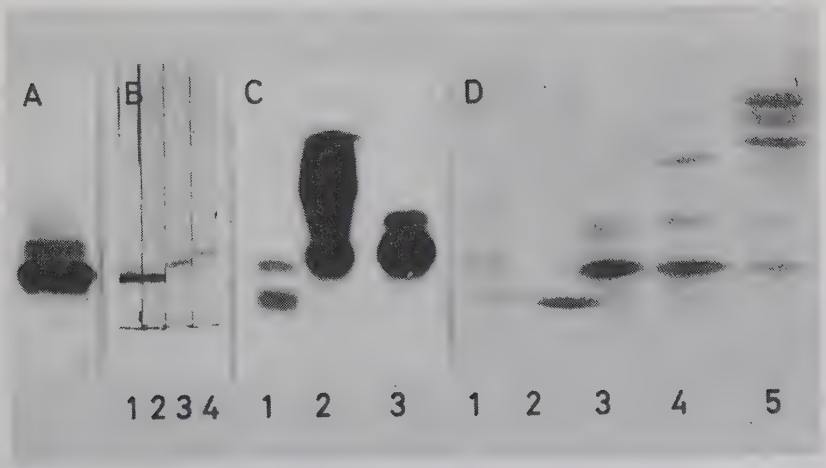
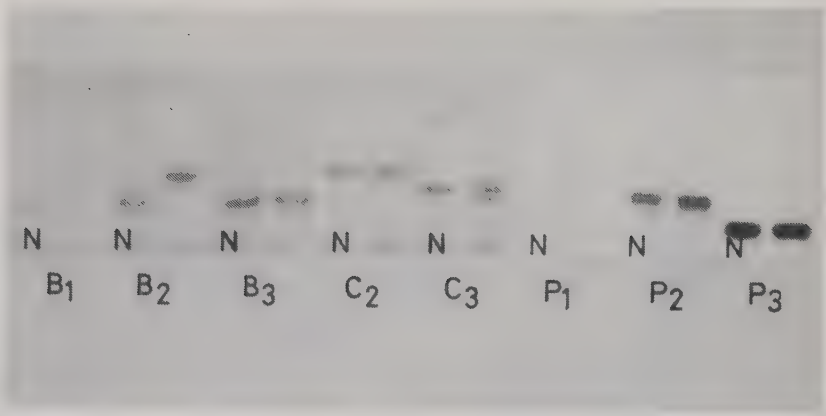


Fig. 3



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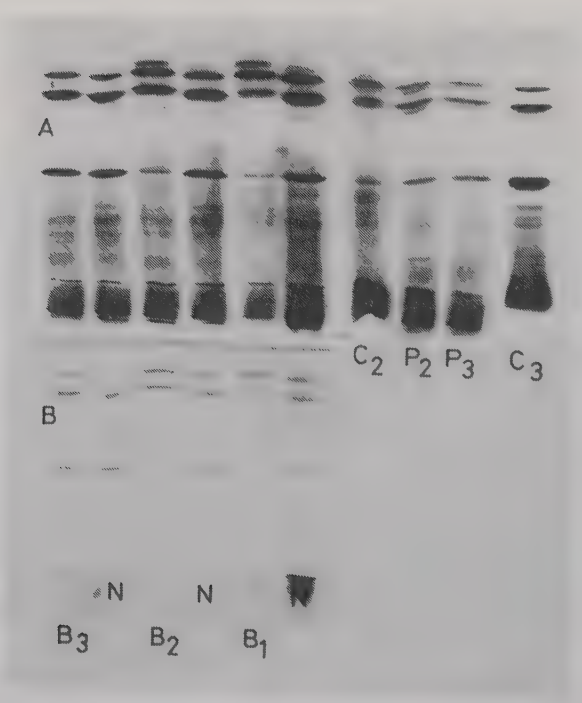

Fig. 5

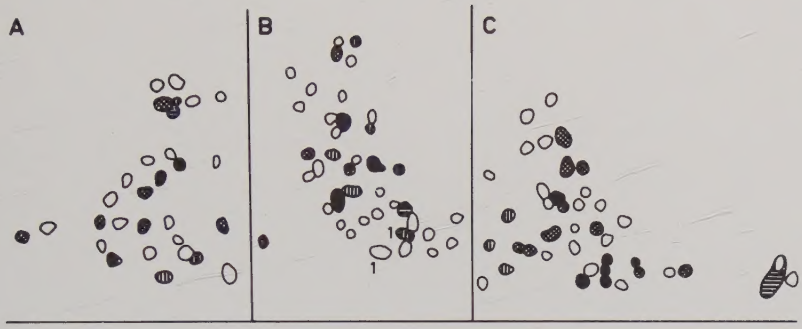

Fig. 6

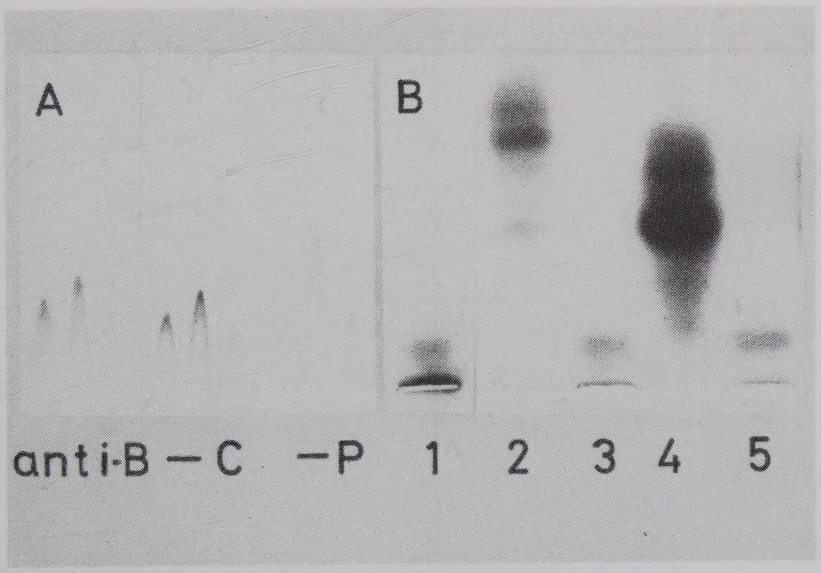

Fig. 7